

A STUDY OF THE CHEMISTRY OF BAC-
TERIAL CELLULAR PROTEINS

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It is the object of this paper to present the results of a study of some of the bacterial cellular proteins from a chemical standpoint. The design has been primarily an investigation of cellular chemistry, especially cellular proteins, but for purposes of comparison other proteins, both animal and vegetable in origin, have been studied. The writer has given special attention to the poisonous group of the proteins though this has been approached by varied methods. After speaking briefly of the preparation of the cellular proteins and of their general reactions, digestion with pepsin and trypsin, the effect of cleavage with dilute mineral acids, and with concentrated acids will be discussed; then the cleavage with dilute alkali, in both water and alcoholic solution, which separates the poisonous from the non-poisonous portion, will be taken up. With only a brief statement of the properties of the non-poisonous portion which has been more closely studied by Leach and Agnew in this laboratory, the chemistry and cleavage of the poisonous part will be presented.

Although much has been done by many investigators upon the varied phases of the chemistry of bacteria little has a direct bearing upon the work here presented, hence for the sake of brevity a résumé of the literature will be omitted at this time.

Preparation of Material.

Both pathogenic and non-pathogenic organisms have been grown and studied. Among these may be mentioned the typhoid

bacillus, colon, anthrax, tubercle, timothy, pyocyaneus, proteus vulgaris, subtilis, ruber of Kiel, megaterium, and sarcina lutea. Other proteins used for comparison have been egg albumen, serum albumen, casein, gelatin, Witte's peptone, Defresne's peptone, De Chapoteaut's peptone, Macquaire's peptone, edestin from hemp seed, zein from maize, also the tissue from two cancers. The bacterial cells in all instances, except the tubercle and timothy bacilli, which were grown on the surface of glycerine beef tea in liter flasks and the anthrax bacillus which was grown on agar in Roux flasks, were grown on 3 per cent agar at room temperature in the large incubating tanks in use in this laboratory. The colon and typhoid need to grow for from ten to fourteen days, but the best growth of the non-pathogenic organisms was obtained in about a week. The thick, slimy, bacterial growth was loosened from the subjacent agar with glass rods, bent twice to form rakes, and then drawn into large flasks by means of suction, from which it was poured into large precipitating jars and thoroughly washed by decantation with 95 per cent alcohol. It was next covered with absolute alcohol and allowed to remain for several hours with frequent stirring, when it was filtered and extracted thoroughly with ether in Soxhlets, after which it was dried and ground in a mortar to a fine powder. The anthrax bacilli underwent similar treatment after removal from the agar of the Roux flasks. With the tubercle and timothy organisms the most abundant growth was obtained at the end of a month. To harvest, the beef tea was decanted through a rather hard filter paper leaving the thick heavy scum of germ growth in the flask, into which absolute alcohol was poured and allowed to remain for some time with frequent shaking to break up the bacterial mass and to thoroughly wash it with alcohol. The whole was next poured onto a hardened filter, and after the alcohol had drained off was placed in Soxhlets, thoroughly extracted with ether, often for several days, or until the ether remained clear, dried and ground to a fine powder. The albumen used was obtained by pouring the whites from twenty dozens eggs into alcohol and after carefully washing with alcohol, extracting thoroughly with ether, drying and pulverizing. The casein and serum albumen were preparations of Kahlbaum and the names of the four peptones indicate their source. The gela-

tin, that used in the laboratory for media, was cut into fine bits and thoroughly extracted with ether in Soxhlets. The edestin was prepared from hemp seed by Leach and Bowles in this laboratory. The ground seed, from which the oil had been expressed, was thoroughly extracted in Soxhlets with either benzene or petroleum ether, and the seeds, freed from extractives, were ground to a powder. From this the edestin was obtained by extracting with a hot 5 per cent solution of sodium chloride. The solution was filtered hot and from it on cooling, the edestin settled out, was filtered, washed and obtained as a fine white powder on grinding. The zein was prepared from corn meal by Leach by extracting with dilute alcohol, precipitating with water, redissolving in alcohol, precipitating with ether, washing, drying and grinding to a powder. The tissue from two cancers, removed by J. W. Vaughan, was cut fine, thoroughly extracted first with alcohol, then with ether, both in Soxhlets, after which each was ground to a powder so far as possible. In these ways all the different materials were obtained in the form of a fine powder varying in color from white to a light brown.

The first work here presented was for the most part with the bacterial cellular substances alone, the comparative work with the other proteins being discussed later in connection with the alkali-alcohol extraction of the poisonous group.

General Properties of the Bacterial Proteins.

The alcohol and ether extractions of the germ substances removed in each case considerable quantities of fats and waxes but these have not been studied to any extent other than to determine that these extracts contained no substance poisonous to animals. The bacterial cellular substance freed from all extractives and in the form of a fine powder as mentioned above was insoluble to any appreciable extent in water or other physical solvents, it being necessary to extract repeatedly with heat as shown by Leach (1) first with dilute sulphuric acid (1 per cent to 5 per cent), then with a solution of sodium hydrate (2 per cent to 4 per cent), to bring it into solution. Physical solvents failed to remove any poisonous body, e. g., a physiological salt solution extract of the colon germ showed a small per cent of

nitrogen but no poison, likewise while water and physiological salt solution extracts of the powdered egg albumen showed faint Millon and biuret reactions with a fair Molisch test, they contained no poison as such but instead sensitized the animals to which they were given.

As was shown by Vaughan and Cooley some years ago the poison contained within the cell of the colon bacillus resists a relatively high temperature. Two hundred milligrams of this substance in 10 cc. of water was put into a tube, sealed and heated to from 180° to 184° for half an hour. This treatment was without appreciable effect on the toxicity. After being heated with water at the temperature of the water-bath for one hour, the colon bacillus retained its form, still took the usual anilin stains, though not quite so deeply and lost none of its toxicity. When thus heated with water the cellular protein does not go into solution and the filtrate from a porcelain filter is inert. The same is true of filtered cultures of the living bacillus, even those twenty days old or older. It is evident that this organism does not produce a soluble poison and that its toxic constituent is intracellular. Heating for from one to two hours in an autoclave at 154° and under two kilos of pressure failed to extract the poison from the bacterial cell. In fact the filtrate from a preparation, heated as just stated, and passed through porcelain gave no cloudiness when dropped into absolute alcohol.

Each of the bacterial cellular substances responded readily to all the protein color reactions including the Molisch carbohydrate test and also reducing Fehling solution after boiling with dilute mineral acid.

In order to compare their relative composition, determinations of ash and of nitrogen by the Kjeldahl method were made as follows, the figures given being the average of two or more determinations.

TABLE 1.

Determinations of Nitrogen and Ash in Germ Substances.

Germ Substance.	Per Cent N.	Per Cent Ash.
Typhoid.....	11.55	5.7
Colon (Leach) (2).....	10.65	8.615
Colon (Agnew) (3).....		8.38
Tuberculosis (Agnew) (3).....	10.55	7.20 (air dried)
	9.27 (air dried)	11.47
Anthrax.....	10.285	9.98 (air dried)
Pneumococcus.....	10.406	7.76
Subtilis.....	5.964	5.78
Proteus vulgaris.....	6.791	10.83
Ruber of Kiel.....	10.655	10.88
Megaterium.....	8.349	6.71
Pyocyaneus.....	10.843	10.18
Violaceus.....	11.765	9.04
Sarcina aurantiaca.....	11.460	6.90
		6.40

As will be seen the nitrogen varied from 5.964 per cent to 11.765 per cent while the ash showed from 5.7 per cent to 11.47 per cent.

Notwithstanding the fact that preparation of the bacterial cellular substances in the form of fine powders as described took away the life of the germs by the thorough extraction with alcohol and ether, the cell still maintained intact its structure under the micr scope and still took anilin dyes perfectly though the tubercle bacillus and the timothy were no longer acid fast. But although the organisms were no longer capable of growth they were all, pathogenic and non-pathogenic, highly poisonous when injected into experimental animals indicating the presence of an intracellular poison. Indeed many of the non-pathogenic germ substances were more poisonous, weight for weight, than that of the pathogenic cells, pointing to the conclusion, suggested by Vaughan, that whether a given bacterium is pathogenic or not depends, not upon the presence or absence of a poison within its complex, but upon whether or not it is able to grow and multiply in the body and likewise to be split up so that its poison is liberated. As the free poison split from the cell will be discussed later the relative toxicity in round numbers of some of the dry, freshly prepared, finely powdered cellular substances,

when injected intraperitoneally into guinea pigs, as determined in this laboratory, may be of interest. The cellular substance is fatal in from 6 to 8 or 10 hours.

TABLE 2.

Relative Toxicity of Dry, Dead Bacterial Cells.

Name of Organism.	Proportion of Cell Substance to Body Weight.
<i>Bacillus anthracis</i> (J. W. Vaughan) (6).....	1 : 1,700
<i>Sarcina lutea</i> (Detweiler) (4).....	1 : 2,030
<i>Bacillus tuberculosis</i> (Wheeler).....	1 : 3,000
<i>Sarcina aurantiaca</i> (Detweiler) (4).....	1 : 25,500
<i>Bacillus violaceus</i> (Detweiler) (4).....	1 : 26,500
<i>Bacillus megaterium</i> (Wheeler).....	1 : 31,000
<i>Bacillus typhosus</i> (Wheeler).....	1 : 40,000
<i>Bacillus pyocyaneus</i> (Wheeler).....	1 : 50,000
<i>Bacillus coli communis</i> (Marshall and Gelston) (5).....	1 : 75,000
<i>Bacillus ruber</i> of Kiel (Wheeler).....	1 : 77,000
<i>Bacillus prodigiosus</i> (Detweiler) (4).....	1 : 90,000

Notwithstanding the fact that the bacterial substances are so poisonous when injected intraperitoneally and that they are also poisonous subcutaneously, when given by mouth they do not affect the health of the animals as is evidenced by the following. A rabbit weighing 3500 grams was kept without food for three days when through a soft tube introduced into its stomach one gram of finely powdered colon germ substance, suspended in water, was forced into the stomach by the use of a syringe. The animal was not visibly affected by this large dose either at the time or subsequently, though something like 50 mg. intraperitoneally would have killed it. More will be said later concerning the effects of the digestive ferments.

Experiment was made to determine whether or not the agent in the animal body which breaks up the germ substances liberating the poison, is diffusible through collodion sacs and whether or not the poison thus set free is itself diffusible and can thus cause death. A collodion sac containing 1 gram of finely powdered typhoid germ substance suspended in 8 cc. of distilled water was inserted into the abdominal cavity of each of two medium sized rabbits about 3 o'clock in the afternoon. The rabbits showed no ill effect that evening from the operation.

At 7 o'clock the next morning No. 1 was found dead; No. 2 still showed no ill effect. The post-mortem of No. 1 showed the sac lying across the transverse colon and around it a very marked edema; the colon was hard, and there were various hemorrhagic spots in the omentum over it. Otherwise there were no abnormal conditions. No. 2 was found dead on the second morning. Autopsy showed a marked peritonitis as is also found after death from the intraperitoneal injection of the dead germ substance. In both cases the collodion sac remained intact after removal from the animal body. It may be inferred therefore that both the germ splitting agent and the separated poison are diffusible through collodion sacs.

Digestion with Pepsin and Trypsin.

The bacterial cellular proteins are, so far as their toxic properties are concerned, quite resistant to the action of pepsin and trypsin. It must be evident that in the study of this subject chemical work must be supplemented by physiological tests. A given sample of the cellular substance of the colon bacillus was tested upon a large number of guinea pigs in order to determine the minimum lethal dose. This was found to be for half grown animals 0.5 mg. given intraabdominally and 1 mg. subcutaneously. This material was then subjected for three days to an artificial gastric juice the efficiency of which was demonstrated simultaneously by its action on coagulated egg white. After three days the soluble and insoluble portions were separated and their toxicity tested. One half milligram of the undigested part given intraabdominally did not kill but 1 mg. did; while 1 mg. given subcutaneously no longer killed but 2 mg. did. Of the part that was dissolved in the acid pepsin solution, doses tested up to 100 mg. had no apparent effect. A like result was obtained from a similar study of the cellular substance of the typhoid bacillus. The amount of cellular substance left undigested after three days exposure to the acid pepsin was about 10 per cent of that originally taken. The conclusion that can be drawn is that the gastric juice slowly digests the bacterial cellular proteins and that in so doing destroys the poisonous group. With trypsin the effect is somewhat different. The cellular protein goes into

solution more rapidly and at least a part of the poison passes into solution without complete loss of its poisonous properties. The parts of both the colon and typhoid cellular proteins that passed into solution after three days' exposure to trypsin killed half grown guinea pigs in doses of from 35 to 40 mg. given intra-abdominally; while the undigested portion killed in doses of from 4 to 7.5 mg.

Cleavage with Dilute Mineral Acids.

Dilute acids slowly break up the cellular protein of the colon bacillus with at most only partial destruction of the poisonous group as is shown by the following:

One hundred milligrams of the cellular substance was boiled with 10 cc. of 1 per cent (by weight) hydrochloric acid. The protein was slightly reddened by this treatment and the mixture on standing separated into a reddish deposit and an opalescent supernatant fluid. The latter was filtered, carefully neutralized with sodium bicarbonate and injected into a guinea pig which died after some hours. In order to study the soluble products obtained by extraction with dilute acid at different temperatures the following experiment abbreviated from an earlier report (18) was made. Two hundred grams of typhoid cellular substance was extracted with 0.1 per cent sulphuric acid in the cold, then the residue at 100° and then at 120°. Four liters of the dilute acid was used at each operation and the extractions at a given temperature repeated so long as any part was removed, when the residue was again treated with acid, and extracted at the next higher temperature. This required three extractions in the cold, four at 100° and two at 120°. The extracts were precipitated with three volumes of 95 per cent alcohol, the precipitate filtered out, washed with alcohol, dried and powdered. The total amount of split product thus recovered amounted to approximately 20 grams or about 10 per cent of the 200 grams of cellular substance, while there were 26.96 grams of the residue finally remaining. The precipitates were purified by dissolving in water, so far as possible and reprecipitating with alcohol. In this way the split products were divided into a soluble and insoluble portion. The different soluble portions from each tempera-

ture were put together and repurified. The insoluble portions were saved except that at 100°. This was very small in amount and so slimy and mucilaginous that it was not possible to remove it from the filter. Determinations of ash, phosphorus and nitrogen were made in both soluble and insoluble portions, and likewise in the final residue and original germ powder. First the ash was determined, then the phosphorus in the ash. The nitrogen in each case was determined by the Kjeldahl method. The figures are given in Table 3.

TABLE 3.

Percentage Composition of Products from Typhoid Germ Substance with 0.1 per cent H₂SO₄.

	COLD.	100°.	120°.	RESIDUE.	GERM.
<i>Soluble:</i>					
Ash.....	72.6	5.28	3.02		
P.....	0.4058	0.385	0.87		
N.....	3.75	10.34	14.5		
N (ash free).....	13.72	10.918	15.01		
<i>Insoluble:</i>					
Ash	55.65		4.78	2.60	5.7
P.....	8.43		1.50	0.21	1.85
N.....	5.89		12.26	13.04	11.55
N (ash free).....	13.28	10.918	12.89	13.424	12.258

It is evident that the extraction in the cold brought down first of all a large part of the inorganic constituents of the germ, among which iron and sodium were found, and it is interesting that the split product which was the most toxic, the soluble part at 120°, was the one containing the largest percentage of nitrogen. These split products were more or less poisonous to guinea pigs but were evidently not the free poison as it took as long a time for them to kill as for the germ substance, that is, from 6 to 8 hours or even longer.

An ultimate analysis was made of the soluble part obtained at 100°, this being the only one of which there was a sufficient quantity for the purpose. The powder was dried to constant weight, and its constituents determined with the results given below. Carbon and hydrogen were determined by combustion, nitrogen by the Kjeldahl method; phosphorus in the ash by

precipitation, first with ammonium molybdate, then with magnesia mixture, sulphur by the alkali method (7) fusion with potassium hydrate and nitrate, solution of the residue in acidified water, and precipitation as barium sulphate, and oxygen by difference.

TABLE 4.
Typhoid Split Product at 100° with 0.1 per cent H₂SO₄.

	C.	H.	N.	S.	P.	O.
With ash (5.28 per cent)	45.73	5.95	10.34	1.13	0.386	36.46
Ash free.	48.27	6.28	10.92	1.45	0.403	32.67

There was also an undetermined amount of iron.

The water solution of this split product gave good tests with the biuret, xanthoproteic and Millon protein reactions. There was also a Molisch reaction, indicating a carbohydrate group which was further supported by the fact that after boiling the substance with dilute acid, Fehling's solution was reduced. The split product was evidently protein in nature, though a compound protein.

It could not have been a protamin for the protamins are much richer in nitrogen, containing 30 per cent or more. A comparison of its percentage composition, calculated ash free, with the percentage composition of ash free animal protein as given by Hammarsten (8) is worthy of note, although a more extensive comparison with most of the known protein substances shows that its composition more closely resembles that of the group of glycoproteins, the mucin substances, especially that of a mucin from sputum, and a mucoid from tendon, as given by Cohnheim (9). The figures are given below. These glycoproteins, however, differ from the split product in that they contain no phosphorus. Only two phosphorized glycoproteins are known (8), namely ichthulin (10), occurring in carp eggs, and helicoprotein (11), obtained from the glands of the snail, and their percentage composition does not compare closely with that of the split product. Ichthulin has sometimes been classed with the nucleo-albumins. These phosphorized glycoproteins, like the split product, yield a reducing substance on boiling with acid.

TABLE 5.

Comparison Percentage Composition of Various Proteins with that of Typhoid Split Product.

	C.	H.	N.	S.	P.	O.	Fe.
Split product (ash free).....	48.27	6.28	10.92	1.45	0.403	32.67	+
Animal protein (ash free).....	50.6— 54.5	6.5— 7.3	15— 17.6	0.3— 2.2	0.42— 0.85	21.5— 23.5	
Mucin from sputum ..	48.26	6.91	10.7	+	—	33.1	
Mucoid from tendon..	48.3	6.44	11.75	0.81	—	32.7	—
Helicoprotein.....	46.99	6.78	6.08	0.62	0.47		+
Ichthulin.....	53.52	7.71	15.64	0.41	0.43		0.1

On the whole the indications are that the split product obtained by the action of dilute mineral acid is of the nature of a phosphorized glycoprotein.

Blandine (12) reported the isolation of a nuclein and nuclealbumin from culture of the typhoid bacillus, while Brieger and Fränkel (13), in their work on toxalbumins, found in cultures of the typhoid bacillus a protein which was toxic for rabbits and which was thought to contain the typhoid toxin in an impure state.

The Nitrogen of Three Acid Extracts. In order to study the distribution and amounts of the protein decomposition products as obtained through the action of acid, it seemed expedient to make a more general study of the nitrogen content of the germ as extracted by acid. To obtain some idea as to the amounts of amino, monoamino, and diamino nitrogen in the decomposition of the cellular substance by acid, represented by ammonia, monoamino acids and the hexon bases and to study their distribution when acids of different strengths were used, a comparative series was made, practically using the method of Hausmann (14). By this method he determined the amino nitrogen, determinable as ammonia, the nitrogen of the diamino bodies, as precipitated by phosphotungstic acid, and that of the monoamino acids as found in the filtrate not precipitated by phosphotungstic acid. The method though severely criticized by Kutschner (15) and others, has still been shown by Osborne and

Harris (16) to give valuable comparative results, whereby general differences are plainly evident. The method of procedure was as follows:

Three 10 gram samples of typhoid germ substance were extracted for five hours on the sand bath with reflux condensers, using 90 cc. of 1 per cent, 5 per cent, and 33½ per cent sulphuric acid, respectively, for the three samples. The residues were each then boiled for an hour with 100 cc. of water and this added to the extract, the whole being then diluted to 200 cc. The ammonia of each extract was determined by distilling with magnesia and the total nitrogen by the Kjeldahl method. The extracts were next each made to contain 5 per cent sulphuric acid, the required amount being added to the 1 per cent, and the 33½ per cent being diluted with the necessary amount of water. After this, each extract was precipitated with 25 per cent phosphotungstic acid, and allowed to stand 24 hours, when the precipitates were filtered out, washed with diluted acidified phosphotungstic acid, dried, weighed and powdered and the nitrogen determined by the Kjeldahl method. The nitrogen was also determined in the filtrates from these precipitates and in the dried residue remaining from the acid extraction. The results are given in Table 6. All the acid extracts gave the xanthoproteic and Millon reactions for protein, and all, save the 33½ per cent, the biuret test. The amount of ammonia nitrogen

TABLE 6.
Nitrogen as. Ammonia-Typhoid.

	1 PER CENT.	5 PER CENT.	33½ PER CENT.
Amount of extract	200 cc.	200 cc.	200 cc.
Wt. N as NH_3 in 200 cc.	0.0112 gms.	0.0112 gms.	0.0112 gms.

Total Nitrogen of Extracts (including N as NH_3).

Amount of extract	200 cc.	200 cc.	200 cc.
Wt. N in extract	0.4284 gms.	0.7644 gms.	1.0164 gms.

Total Nitrogen Separated as NH_3 .

	2.6143	1.4652	1.1019
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split off was the same in each case, which pointed to a definite cleavage regardless of the strength of the acid. The percentages varied, of course, since the total nitrogen of the extracts was different. The largest amount of total nitrogen was found in the $33\frac{1}{3}$ per cent extract, and hence the smallest per cent of ammonia nitrogen, while the smallest total nitrogen was in the 1 per cent extract. The ammonia nitrogen varied from 1.1 per cent to 2.6 per cent. Hausmann (14) found approximately 1.2 per cent ammonia nitrogen in true proteins, which was in accordance with the results of other investigators.

TABLE 7.

Calculated for Typhoid Germ Substance (10 grams sample used).

	Per Cent
Total nitrogen.....	11.55
Ammonia nitrogen.....	0.112
Per cent of total N of germ as ammonia nitrogen.....	0.97

The total nitrogen of the germ was determined by the Kjeldahl method applied to samples of germ powder. The per cent of ammonia nitrogen was found from the fact that 0.0112 gram nitrogen was obtained from each of the three extracts from 10 gram samples.

TABLE 8.

Nitrogen of the Phosphotungstic Precipitates.

	1 PER CENT.	5 PER CENT.	$33\frac{1}{3}$ PER CENT.
Wt. ppt.....	7.8220	12.8602	16.9792
Wt. N in ppt.....	0.2014	0.3429	0.2029
Per cent N in ppt.....	2.575	2.667	1.197

The largest amount of diamino nitrogen was found in the precipitate from the 5 per cent extract. It is probable that the $33\frac{1}{3}$ per cent acid was strong enough to further decompose the diamino compounds and so lessen their quantity. In working for hexon bases a 5 per cent acid extract might be expected to produce the largest yield. The percentages of nitrogen in the precipitates from the 1 per cent and 5 per cent extracts were notably close together, and it is of interest that the 2.66 per cent nitrogen

found in the precipitate from the 5 per cent extract was quite close to the 2.68 per cent nitrogen calculated by Gulewitsch (17) in the phosphotungstic precipitate of arginin, but the carbon and hydrogen found were about twice what he calculated. His figures were C, 3.44 per cent; H, 0.74 per cent, while this precipitate contained C, 8.34 per cent, and H, 1.68 per cent.

TABLE 9.
Nitrogen of Filtrates from Phosphotungstic Precipitates.

	1 PER CENT.	5 PER CENT.	33½ PER CENT.
Total amt. of filtrate.....	200 cc.	260 cc.	260 cc.
Wt. N in whole filtrate.....	0.0315 gm.	0.07189 gm.	0.36218 gm.

The largest amount of monoamino nitrogen was found in the filtrate from the 33½ per cent extract; the smallest in that from the 1 per cent, showing that the weaker acids do not produce so complete cleavage as the stronger.

TABLE 10.
Nitrogen of Residue Left from the Acids.

	1 PER CENT.	5 PER CENT.	33½ PER CENT.
Wt. residue from 10 gms. germ substance..	3.9630	1.7322	0.7308
Wt. N in residue.....	0.4633	0.1924	0.0457
Per cent N in residue.....	11.6900	11.1064	6.2631

The per cent of nitrogen in the residues from the 1 per cent and 5 per cent acids was not greatly changed from that of the original typhoid germ substance (11.55 per cent), which would seem to indicate that the residue was still undecomposed.

Thus it will be seen that dilute acids slowly break up the cellular proteins; that about 10 per cent of the weight of the original germ substance may be recovered as split product; that the split product seems to be of the nature of a phosphorized glycoprotein; and that while the poison of the germ substance seems to be transferred in part, at least, in these split products, it can by no means be regarded as existing in anything but a combined

state as it still requires from 6 to 8 hours to kill an animal just as in the case of the cellular substance. Furthermore it appears that the acid cleavage of ammonia nitrogen from the germ powder of the typhoid bacillus is definite; that the largest amount of diamino nitrogen is obtained by the use of 5 per cent acid as compared with that obtained by the use of 1 per cent and 33½ per cent while the largest amount of monoamino nitrogen is found in the 33½ per cent acid extract.

Cleavage of Cellular Substance with Strong Mineral Acid.

Investigators thus far have looked very largely to a study of the decomposition products of the various albumins to gain an insight into their structural composition. The evident protein nature of the bacterial cellular substances leads to the inquiry as to whether or not the decomposition products of these germ complexes would correspond to those from better known proteins. Some light has been thrown upon the question by work done in this laboratory. Search has been made especially for two groups of bodies which have come to be regarded as essential constituents of all true albumins, namely the hexon bases and monoamino acids. Leach (1 and 2) has established the presence of lysin in the colon bacillus and shown the possibility of both histidin and arginin. Agnew (3) has obtained from the germ substance of both colon and tubercle bacilli monoamino acids as follows:

	COLON. Per Cent.	TUBERCULOSIS. Per Cent.
Glutamic acid.....	3.00	0.2
Glycocoll.....	0.33	
Alanin.....	1.00	1.40
Valin.....	1.60	4.60
Leucin.....	2.00	1.82
Phenylalanin.....	0.20	0.50

Leach in one experiment extracted about 300 grams colon germ substance with 33½ per cent sulphuric acid and worked up the extract for hexon bases by the Kossel and Kutscher method (19) leaving a phosphotungstic filtrate to be investigated for monoamino acids. This was turned over to the writer and from

it both tyrosin and leucin were obtained as follows. From the solution, phosphotungstic acid was removed with barium hydrate and carbon dioxid used to remove excess of barium. By concentration and crystallization bodies were obtained resembling tyrosin and leucin under the microscope. These were purified by repeated recrystallization from water or from ammoniacal water, the tyrosin being so much less soluble than the leucin that they could be separated by difference of solubility. It was necessary to boil the leucin fraction with animal charcoal to remove coloring matter. The tyrosin formed the characteristic colorless silky needles, many grouped in the characteristic sheaves. As it became more pure the needles were longer and larger and grouped in the sheaves less persistently. After many purifications the crystals melted at a constant temperature, though this was difficult to determine since tyrosin melts with decomposition. The melting point maintained after each of two or three recrystallizations was 288° uncorrected. The correction was 8.13° which made the corrected point 296.13° . A Kahlbaum preparation of tyrosin in the laboratory melted within a degree of the same point and agreed in the chemical tests to be mentioned presently. Richter gives the melting point of tyrosin as 235° , Cohn as 295° , while Fischer says that with rapid heating the corrected point is 314° to 318° . The tyrosin obtained gives a Hofmann's test with Millon's reagent. It gives Scherer's test with nitric acid and sodium hydrate on platinum foil and also a beautiful Piria test with sulphuric acid, then barium carbonate and ferric chloride, which test is characteristic for tyrosin.

The leucin crystallized in the characteristic knobs or balls. As it became purer it crystallized more and more in shining white very thin plates, sometimes in radial groups, sometimes not. The crystals were finally obtained with a practically constant melting point 262° to 263° or corrected 268.6° to 269.6° . The pure laboratory leucin (Kahlbaum) melted at the same point. Schwanert, Hammarsten and others give the melting point for active leucin as 170° . That for the inactive form is given as 270° . Fischer says the melting point is 293° to 295° (corrected) if heated quickly in a closed tube. Cohn gives 275° to 276° . The leucin obtained melted with darkening and decomposition. With careful heating in an open tube it sublimed with the characteris-

tic white, wooly deposit. It also responded to Scherer's test on platinum foil with nitric acid and sodium hydrate which test Hammarsten says is characteristic for leucin.

Cleavage of the bacterial cellular substances with strong mineral acids, yields then, both monoamino and diamino acids, thus giving another proof of true protein composition.

Cleavage with Alkali in Water Solution.

With dilute alkali in the cold, 0.5 per cent potassium hydrate with the colon bacillus and both 0.5 and 5 per cent potassium hydrate with *sarcina lutea*, very little is removed from the cell as has already been shown by the writer (20). Heat is necessary to effectively split the molecular structure. When heated with dilute alkali the extracts obtained are thick, syrupy liquids containing a deposit of a slimy, mucilaginous nature which makes filtration exceedingly difficult, the method proving most satisfactory being the use of several thicknesses of hardened paper without the pump. These water alkaline extracts give all the protein color reactions, including a strong Molisch carbohydrate test and the reduction of Fehling's solution after boiling with dilute mineral acid. Excess of alkali or inorganic salt often interferes with the Millon test which is obtained perfectly upon removal of the alkali or upon sufficient dilution.

In one instance the colon germ substance from six incubating tanks, about 300 grams, was heated on a boiling water-bath with 6 liters of a 2 per cent potassium hydrate solution, the extract filtered through folded papers, and acidified with acetic acid. The precipitate produced, presumably protein, and filtered out after standing twenty-four hours was so small in amount that it was lost in the filter paper. Three volumes of 95 per cent alcohol for every volume of acidified extract were then acidified with hydrochloric acid to the amount of 0.5 per cent and into this acid alcohol the acidified extract was poured, producing a heavy white, curdy, fibrous precipitate, which was filtered, washed acid free with alcohol and then washed with ether. After purification by solution in one liter of 0.5 per cent potassium hydrate, reprecipitation in three liters 95 per cent alcohol, washing as before with alcohol and ether,

there remained 25 grams of the white, flaky precipitate, something less than 10 per cent of the original germ powder. Injections of solutions of this precipitate up to 125 mg. had no effect upon guinea pigs showing that the poison was not there. The possibility of having obtained a nuclein or nucleic acid naturally suggested itself. Determination of ash and phosphorus showed 5.9 per cent of ash and 0.194 per cent of phosphorus. Among the protein color reactions this split body gave the xanthoproteic alone and that none too well. With α -naphthol and sulphuric acid it gave a beautiful furfural ring (Molisch carbohydrate test) and when boiled with dilute mineral acid it reduced Fehling's solution with great readiness. The low percentage of phosphorus showed that it could not be a nuclein or nucleic acid while the other tests pointed to the presence of a carbohydrate. To try the possibility of further splitting the body a 1 per cent (by weight) sulphuric acid extract was made in the cold, the extract filtered and precipitated with 95 per cent alcohol. It was thought that there might thus be obtained a precipitate with a higher phosphorus content. Instead, the three parts of the first precipitate, that is, the precipitate and filtrate of the acid extract and the part remaining from the acid extraction, gave only carbohydrate tests. As the primary search was for the poison this carbohydrate was not identified at this time.

From the first alcoholic filtrate from this carbohydrate, in other words, from the alcohol soluble part of the 2 per cent potassium hydrate extract of the colon germ substance, there was obtained, after evaporation of all liquid, resolution in absolute alcohol to remove inorganic salts and evaporation of the alcohol, a black, gummy, sticky, viscous mass which defied every attempt at purification and every effort to put it into a solid, weighable form, but which gave perfectly the protein color reactions. In this black, sticky mass, containing apparently a protein soluble in absolute alcohol, was found also a poison, small unweighed doses killing animals in 6 or 8 minutes, which was quite different from the 6 or 8 hours necessary to a fatal issue with the cellular substance and which indicated the poison in a more free or less combined state so that its physiological effect was obtained at once without the time interval necessary for the body juices to act upon the complex structure of the

cellular molecule and thus to liberate the poison. Various efforts to extract the poison from its viscous host with ether, chloroform, petroleum ether, amyl alcohol, etc., were tried to no avail. Ammonium sulphate, to saturation, salted out only a very little protein and produced no separation. Mercuric chloride and lead acetate gave excellent precipitates but in each case, after removal of mercury and lead from their respective precipitates and filtrates, the poison remained always in the filtrates. Phosphotungstic and phosphomolybdic acids produced precipitates but no other indication of alkaloidal bodies. It seemed necessary for further progress to obtain the poisonous portion in a state of greater purity and if possible free from the sticky carbohydrate. Hydrolysis with dilute sodium hydrate in both water and absolute alcohol solution was tried, sodium being used to replace potassium in order to avoid the possible presence of poisonous inorganic salts. By extracting with 1 per cent alkali, no poison was obtained in either water or alcohol, but when the sodium hydrate was increased to 2 per cent as before, both water and alcoholic solutions contained a highly poisonous body. The decided advantage however lay with the alkali-alcohol extraction, in that by this method a larger proportion of toxic material was obtained; it was possible to get rid of most of the inorganic constituents by simply acidifying with hydrochloric acid and filtering out the sodium chloride precipitated in the alcohol and lastly all of the carbohydrate remained in the insoluble residue so that by careful distillation and evaporation, both in vacuo, of the alcohol from the neutralized extract, the poisonous substance was obtained in a hard brittle mass which could be ground to a fine powder. The poisonous portion of the cellular substance as extracted by dilute alkali in absolute alcohol will be discussed in detail after mention is made of the effect of strong alkali in water.

Strong alkali was used to find out whether or not it would separate the poison in a still simpler or less combined form. Colon germ substance was boiled on the sand-bath for four hours with strong sodium hydrate (1:2). During the process large quantities of ammonia were given off. At the end of this time the black, rather thick solution was diluted with an equal volume of water and filtered. By far the larger part

of the cellular protein had gone into solution, only a small residue remaining. The clear filtrate was acidified with hydrochloric acid, giving off a strong indol odor though no test for indol could be obtained. The solution was next evaporated to dryness. The dry substance gave no biuret test, but still showed the Millon and Molisch reactions. It burned with practically no ash and contained both nitrogen and sulphur, the presence of phosphorus being doubtful. A portion of this dry substance, extracted with absolute alcohol, filtered, evaporated, redissolved in water and neutralized with sodium bicarbonate was somewhat poisonous to guinea pigs but not highly so, indicating that the poisonous action was diminished rather than increased by the use of strong alkali.

Cleavage with Dilute Alkali in Alcohol.

The first work upon the cleavage with dilute alkali in alcohol was done with the bacterial cellular proteins but later the method was applied to other proteins of both animal and vegetable origin. The process, which is the same for any protein, has already been described in detail in a previous paper (21), having been in use in this laboratory for several years, but may be outlined for the sake of clearness. The dry cellular substance or the protein from whatever source, prepared as has been described in the form of a fine powder, is boiled for an hour on the water-bath, under a reflux condenser, with from fifteen to twenty-five times its weight of absolute alcohol, in which 2 per cent by weight of pure sodium hydrate has been previously dissolved. It is essential that the alcohol used in this extraction should be absolute, and that every trace of water in the substance should be removed before the extraction of the poison is undertaken. If this point be neglected the poison will be obtained in the same sticky, gummy mass as with the water-alkali extraction and with which satisfactory results are impossible. By this process the germ substance or the animal or vegetable protein is divided into two distinct parts. From the golden-brown alcoholic solution there is obtained a powder representing about one-third of the original dry germ substance and a somewhat larger proportion of the other proteins, which is

very toxic, killing guinea pigs in small doses in about thirty minutes, while the residue insoluble in alcohol, represents about two-thirds or less of the weight of the original powder according as to whether obtained from cellular substance or from a more simple protein, and is not poisonous. The protein does not yield all its poison, as a rule, until the extraction with sodium hydrate in alcohol has been repeated at least three times. Boiling the bacterial, animal or vegetable protein thus with sodium hydrate in alcohol splits the molecule along certain definite lines of cleavage, for when the extraction has been repeated several times, boiling for an hour at each extraction, it will be found that all the poison is dissolved in the alcohol, that no more can be split off, and that the portion insoluble in alcohol has no longer toxic properties. After these portions have been separated by filtration, the filtrate, containing the poison dissolved in alkaline absolute alcohol, is neutralized with a calculated amount of hydrochloric acid. This throws down practically all the base and acid as sodium chlorid, which is removed by filtration. There is thus obtained a solution of the poisonous part of the germ cell or other protein in absolute alcohol. The greater part of the alcohol is distilled in vacuo under 40° and the evaporation continued to dryness in vacuo at 40° . Resolution in absolute alcohol removes remaining traces of sodium chlorid, the solution being again evaporated to dryness in vacuo at 40° . The poisonous part of the protein obtained in this way provided there is no water present, forms a brown scale on the dish, which may be pulverized to a brownish powder that is toxic in small doses.

It is believed that this poisonous substance is an integral part of the bacterial cell or other protein, hydrolyzed from it by the alkali, and that it is a synthetic product formed during the protein's growth. If the poison is not obtained from the germ cell, the only other source possible is from some part of the culture medium. In this case it would have to come either from the beef tea or from the agar. To test the possibility, 200 cc. of beef tea were poured into five volumes absolute alcohol. After settling, the precipitate was filtered out, dried and weighed. There were 1.5 grams. Ordinarily, when beef tea cultures are employed to inoculate the tanks from 500 to 600 cc. are used.

This would mean that if all the beef tea remained at the harvest and were removed with the bacterial growth that there would be a possibility of precipitating by the alcohol along with from 200 to 300 grams of germ substance, at most from 3.75 to 4.5 grams of foreign substance. As a matter of fact it must be very much less, the beef tea being exhausted by the growth of the germ, so that it will readily be seen that this source could not yield some 70 to 100 grams of poison. As to any possibility of agar as a source of the poison, 40 grams of agar were cut very fine, extracted on the water bath with 2 per cent sodium hydrate in absolute alcohol, dried, powdered in a mortar, and reextracted with sodium hydrate in absolute alcohol. The yellow alkaline extracts, filtered from the insoluble residue, were just acidified with hydrochloric acid, the salt filtered out, and the clear solution evaporated to dryness. The dry residue was very small in amount, mostly insoluble in water, showed none of the protein color reactions, and had no effect whatever upon animals, thus eliminating agar as a source of error. On the positive side of the question to show that the poison is an integral part of the cell and built up synthetically during its growth from the elements of the cultural medium, the colon germ was grown on Fraenkel's modification of Uschinsky's medium, which contains no protein whatever, as such. After a week's development the contents of the flask were poured into from two to three volumes of 95 per cent alcohol, the precipitated substance filtered out and put into absolute alcohol. Filtered from this it was extracted in Soxhlets with ether, dried and powdered. From this germ substance the process for the extraction of a poisonous body with 2 per cent sodium hydrate in absolute alcohol was carried out as has been outlined. On concentration of the alcoholic solution there was obtained a poisonous body just as from the extraction of germ substance from agar grown cultures. The same dose killed guinea pigs in thirty minutes, with characteristic symptoms of colon poison. Also, the poison obtained from germ grown on Fraenkel's modification of Uschinsky's medium gave all the protein color tests described later as being obtained from colon poison from cultures grown on agar. This demonstrates that the poison is an integral part of the germ split off from the cell by alkali, and in consideration of its apparent

protein nature, set forth in detail subsequently, it is evident that the bacterial cell must synthetically produce this protein body during its growth from the chemical components of the medium.

Considering the animal and vegetable proteins, aside from those of bacterial origin, it is evident that here the bodies are obtained in a purer and more simple form and that what is split off by the alkali must be produced from the substance itself. Also it seems logical to conclude that the line of cleavage obtained by the use of alcoholic alkali must somewhat nearly approximate the physiological process, since after sensitization, an injection of the whole protein kills with precisely the same symptoms as are obtained with a fatal dose of the split off poison.

Ammonia Set Free by Cleavage with Dilute Alkali. During the extraction of the protein poison with dilute alkali the odor of ammonia is apparent even at the end of the third extraction. An effort was made to discover how much nitrogen was converted into ammonia. A device was arranged for conducting the ammonia into standard acid, $\frac{N}{10}$ sulphuric being used, and four ten gram samples of Witte peptone were extracted with 2 per cent sodium hydrate in absolute alcohol, one for 3 hours in a current of air the other three in a current of hydrogen for 2.5, 8.5 and 19.5 hours respectively. At the end of each operation the excess of acid was titrated with $\frac{N}{10}$ sodium hydrate and the per cent of nitrogen calculated. The toxicity of the split product was also determined. In every case ammonia was still being produced when the process was interrupted. Again, a ten gram sample of albumin poison was boiled for 54.5 hours with the 2 per cent alcoholic alkali to find out whether ammonia could be split from the poison itself and the effect. The table below shows the results.

TABLE 11.

Ammonia Produced by Cleavage of Protein with Dilute Alkali in Absolute Alcohol.

SAMPLE.	TIME IN HOURS.	ATMOSPHERE.	PER CENT N OF SAMPLE GIVEN OFF.	RATE N PER HOUR IN PER CENT.	TOXICITY OF POISON.
Witte Peptone.....	3	air	0.4305	0.1435	Diminished.
Witte Peptone.....	2.5	H	0.3956	0.1582	Increased above that extracted in air.
Witte Peptone.....	8.5	H	0.7383	0.0868	Between that of the first two above.
Witte Peptone.....	19.5	H	1.0517	0.0539	
Albumen Toxin.....	54.5	H	1.48	0.027	Diminished by half.

Albumin toxin as ordinarily extracted contains 13.74 per cent of nitrogen. By the 54.5 hours heating with alcoholic alkali 10.77 per cent of its nitrogen was converted into ammonia. After this treatment the poison still gave a good Millon test but no longer a biuret. It is probable that by continued heating in the same manner quite all of the nitrogen could be separated though it is noticeable that the rate per hour was gradually diminished as the time lengthened.

Properties of the Non-Poisonous Portion.

It is not within the province of this paper to speak at length of the non-poisonous portion of the protein molecule, a number of papers having already appeared upon the subject from this laboratory, from its chemical standpoint by Leach (22) and Agnew (3), from its physiological side by Vaughan (23), V. C. Vaughan, Jr. (24), J. W. Vaughan (25) and by Vaughan and Wheeler (26) jointly. Suffice it to say that the non-poisonous portion contains the specific part of the protein, the part that immunizes and sensitizes. It gives the protein color reactions, the Millon test rather poorly; it contains most of the phosphorus and all of the carbohydrate. Percentages of nitrogen and ash

in a number of these residues, determined by Gidley and Houser in this laboratory, not published elsewhere, save two by Leach, may be of interest.

TABLE 12.
Nitrogen and Ash in Non-poisonous Portion of Proteins.

RESIDUE.	PER CENT N.	PER CENT ASH.	PER CENT N ASH FREE.
Colon (Leach).....	5.56	26.08	7.52
Tuberculosis.....	7.295	23.36	9.53
Typhoid.....	5.955	16.50	7.13
Anthrax.....	2.43	46.73	4.56
Pyocyaneus.....	7.29	27.25	10.02
Proteus Vulgaris.....	5.99	25.29	8.02
Ruber of Kiel.....	5.58	15.49	6.60
Subtilis.....	7.00	21.63	8.93
Megaterium.....	4.855	18.50	5.95
Albumen (Leach).....	12.67	13.57	14.53
Casein.....	8.73	19.80	10.88
Edestin.....	13.125	15.19	15.48
Serum Albumen.....	6.995	32.27	10.26
Witte Peptone.....	10.38	19.90	12.98
Macquaire Peptone.....	7.185	32.73	10.63
De Chapoteaut Peptone.....	6.78	41.46	11.58

A large part of the ash is composed of inorganic salts due to presence of the sodium hydrate which can be completely removed from the residue only by dialysis.

General Properties of the Poisonous Portion.

The poison split from the protein molecule by hydrolysis with dilute sodium hydrate in absolute alcohol is undoubtedly crude. It is probable, as the ammonia determinations show, that the process by which it is obtained serves also to destroy or diminish it if carried too far. But crude as it is, there is present a poisonous body, fatal to guinea pigs intraperitoneally in doses of from 8 to 60 mg. according to its purity and in from 30 to 60 minutes, or even in shorter time if the dose be large, and the symptoms produced are identical in kind, in sequence and relative duration whatever the protein from which the poison is obtained. The

physiological action of the poison has been presented by V. C. Vaughan, Jr. (27), and will not be further discussed here except to say that by mouth it is not poisonous, a fasting rabbit bearing a whole gram of typhoid toxin introduced into its stomach by means of a tube, without apparent effect.

So far a large number of protein bodies bacterial, animal, vegetable, have been split up and no true protein as yet has failed to yield a poisonous group. Among the poisons obtained are those from *Bacillus coli communis*, *Bacillus typhosus*, *Bacillus anthracis*, *Bacillus tuberculosis*, *Bacillus mœllerii* (timothy), *Sarcina lutea*, *Bacillus ruber* of Kiel, *Bacillus proteus vulgaris*, *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus pyocyaneus*, from egg albumen, casein, serum albumen, edestin, zein, Witte peptone, Macquaire peptone, De Chapoteaut peptone and from the tissue of two cancers. Gelatin contains no poison, but gelatin is an albuminoid, and scarcely gives the Millon test, if at all. Witte's peptone contains a poison as does also that of Macquaire and De Chapoteaut, but Nicolle and Abt (28) have found that Defresne's peptone does not and this we have confirmed. However, we do not know whether this is made from gelatin or from a true protein.

The brownish, poisonous powder is very hygroscopic and dissolves freely in water, forming an opalescent solution, acid in reaction, and pale yellow to golden brown in color according to its strength. The opalescence is removed by filtration through a hardened paper, but the toxicity of the solution is not thereby diminished, showing that a small but non-poisonous part of the powder is insoluble in water, but the larger part is so freely soluble that for practical purposes the opalescence may be disregarded. On neutralizing the opalescent solution with sodium hydrate it becomes clear; on neutralizing with sodium bicarbonate, a brownish, non-toxic precipitate is formed, small in amount, and when this is removed by filtration the solution obtained is clear and highly toxic. In absolute alcohol the solution is perfectly clear. After long standing in an absolute alcohol solution, there is a small part that settles out and becomes insoluble; also, after repeated evaporation to dryness and resolution in absolute alcohol, a small part each time becomes insoluble. The powder is soluble in methyl as well as ethyl alcohol but not in amyl

alcohol, and is insoluble in ether, chloroform and petroleum ether. Each of these latter will remove a very small amount of fatty substance which is non-toxic, but they do not dissolve an appreciable amount of the poison. The powder is soluble in strong mineral acids remaining clear on being boiled and on diluting with water. A few drops of mineral acid, however, added to a water solution, causes a precipitate, which would seem to indicate that the acidity of the water solution of the poison is caused by the presence of some organic acid.

The poison diffuses slowly through collodion sacs both within the animal body and suspended in distilled water, as is shown by the following. Two hundred milligrams of typhoid poison dissolved in 20 cc. of water was placed in each of two collodion sacs which were then suspended in distilled water. At the end of 24 hours the Millon reaction was given by the dialysate. This was replaced every 24 hours by distilled water and the dialysis continued for 96 hours. At the end of this time the combined dialysates were concentrated to dryness, dissolved in absolute alcohol and again evaporated to dryness. The brown residue, sticky in appearance, dissolved in water was acid in reaction, had the characteristic "toxin odor," and killed a guinea pig in 20 minutes showing that the poison is diffusible. So slowly, however, does it diffuse that even at the end of the 96 hours it was not yet entirely removed from within the sac. In another experiment a collodion sac containing one gram of typhoid toxin suspended in 8 cc. of water was put into the abdominal cavity of a medium sized rabbit. There resulted no visible effects upon the animal. After 12 days the sac was removed. In the sac, which was intact, were found 6 cubic centimeters of a clear fluid, which looked more like blood serum than anything else. Three cubic centimeters of this fluid had not the slightest effect upon a guinea pig showing that the toxin had undoubtedly diffused but had evidently diffused so slowly that there was not enough present at any one time to affect the health of the rabbit.

Although the toxic powder is so readily soluble in absolute alcohol, the tests point to the presence of an albuminous or protein body. Practically all the protein color reactions are obtained, with the exception of Molisch's test. It is interesting,

too, that the part separating out from an alcoholic solution on long standing gives none of the protein color reaction, indicating that the protein part is permanently soluble in absolute alcohol. The Millon's reaction shows most perfectly and persistently wherever the poison is found. Mann (29) says that this reaction is given by all benzene derivatives in which one hydrogen atom has been replaced by the hydroxyl group OH and as, according to Salkowski (30) and Nasse (31), tyrosin is the only oxyphenyl compound in protein, the reaction is presumed to show the presence of tyrosin. The biuret reaction, not obtainable with any but the albuminous dissociation-products of protein and used to distinguish between albumins and their simpler or secondary decomposition products, is given most beautifully. Heated with strong nitric acid a clear pale yellow solution is formed, which when ammonia to excess is added, turns to a deep orange color, thus giving the xanthoproteic test and indicating the presence of aromatic radicals. The reaction of Adamkiewicz is positive, for when the poison is heated with acetic acid or, as Hopkins and Cole (32) suggest, shaken with glyoxylic acid, and then strong sulphuric acid is added, a beautiful violet color develops. Hopkins and Cole (33) have shown that this reaction depends on tryptophan or indol-amino-propionic acid. On boiling with concentrated hydrochloric acid, to which a drop of concentrated sulphuric acid has been added, the powder passes into solution and a violet color results, thus giving Lieberman's test, which was considered by Hofmeister (34) to be a furfural reaction in which furfural produced from the carbohydrate radical of the albumen molecule and the aromatic oxyphenyl radical took part, though Cole (35) asserts that it is due "to an interaction between the glyoxylic acid which is present in the ether used for washing the albumin, and the tryptophane which is split off from the albumin by the action of concentrated hydrochloric acid." However this may be the poison does not respond to the Molisch test, given by the formation of furfural on the splitting up of a carbohydrate group with sulphuric acid, nor does it reduce Fehling's solution either directly or after boiling with dilute mineral acid.

The ordinary test for sulphur in proteins, that of heating with excess of sodium hydrate in the presence of a small amount of

acetate of lead, is not given by the portion of the protein split off by alkali in absolute alcohol. If, however, a portion of the substance in a test tube is fused with metallic sodium and the cooled mass treated with water, a few drops of a freshly prepared solution of sodium nitroprussiate added to part of the clear filtrate give a beautiful violet color, indicating the presence of sulphur. Also, if the other part of the clear filtrate be treated with a lead acetate solution and acidified with acetic acid, lead sulphid is precipitated. If the solution is acidified before lead acetate is added a faint but unmistakable odor of hydrogen sulphid is detected. It is known that sulphur may exist in the albuminous molecule in at least two forms, one part being readily split off on heating with dilute alkalies as hydrogen sulphid, the other being obtained only when the destruction of the protein molecule is carried much farther. Since the nitroprussiate reaction is very delicate, no conclusion as to the amount of sulphur can be drawn from this test, and although a good precipitate of lead sulphid is formed the amount of sulphur is probably not large, since Leach failed entirely to find sulphur present in the ash of the colon bacillus, though both the germ substance and the residue which remains after extraction of the poison, respond to the nitroprussiate test for sulphur, and also give the lead sulphid precipitate in the clear acidified filtrate from the fused mass.

A solution of the toxic substance is not coagulated by heat in acid, neutral or alkaline solution, though, as already stated, a few drops of a mineral acid causes the appearance of a considerable precipitate which is not soluble on heating or on the further addition of acid. This precipitate is produced regardless of whether or not the opalescence of the water solution is first removed.

Among the metallic salts, copper sulphate produces no precipitate and ferric chloride only on heating. Silver nitrate naturally precipitates any trace of chlorid present, but after the addition of an excess of ammonia there still remains a small precipitate. Potassium ferrocyanid gives a precipitate, also potassium bismuth iodid in acid solution. Lead acetate, mercuric chlorid and platinum chlorid all produce heavy precipitates. With lead acetate and mercuric chlorid, however, after removal

of lead and mercury with hydrogen sulphid from their respective precipitates and filtrates, the protein reactions are given by the filtrates and here also is found the poison in each case. From 10 to 15 per cent of the crude poison can be precipitated by the use of platinum chlorid in either water or alcoholic solution. All attempts to crystallize this precipitate failed as only a small part of it is dissolved by hot water and the insoluble part is unaffected by any of the other ordinary solvents. The protein reactions are given by the platinum precipitate by both soluble and insoluble parts, but not by the filtrate. The poison is found in the insoluble part of the precipitate after removal of the platinum by hydrogen sulphid, its toxicity being markedly increased. The other parts, after removal of the platinum are inert.

From a water solution of the poison, bodies giving protein reactions may be salted out by the addition of ammonium sulphate or sodium chlorid to saturation, but in neither case is the separation complete, the filtrate still responding to the protein color tests after removal of the neutral salt. In the case of salting out with ammonium sulphate, the solubility of both parts is thereby lessened and the toxicity diminished, possibly on account of decreased solubility, though both parts exhibit some poisonous action and likewise both show the protein color tests.

Phosphotungstic, phosphomolybdic and picric acids all give abundant precipitates. Since these reagents are also used in the precipitation of alkaloidal bodies, the precipitates with phosphomolybdic and phosphotungstic acids were further examined, the possibility suggesting itself that the toxic body might be alkaloidal in nature and that the protein part might be entirely separate from the poison. A sample was precipitated with phosphomolybdic acid in acid solution, the precipitate removed, washed and dissolved in ammoniacal water. This solution was then shaken with amyl alcohol but the alcohol was not colored and the residue obtained on concentration was so slight as to be practically nothing. Another sample was precipitated with phosphotungstic acid, the solution being acid in reaction. The precipitate was allowed to settle, removed by filtration, washed with acidulated water, decomposed with a saturated solution of barium hydrate and the remaining insoluble part filtered out. So far

as possible the barium was removed from the filtrate with carbon dioxid, alternating with concentration and further addition of carbon dioxid. The solution was then allowed to concentrate to dryness, when the residue was dissolved in absolute alcohol, leaving barium salts behind.

On concentrating the slightly opalescent solution, more barium salts came down during the process and were filtered out. The dry residue was taken up in water and ammonium carbonate used to precipitate the barium that still remained. After removing the barium carbonate, by evaporating on the water bath, both carbon dioxid and ammonia were expelled, the solution again becoming acid. Dryness being reached absolute alcohol was once more used, leaving undissolved a small amount of inorganic material. In this way the final residue after evaporation of the alcohol was practically freed from inorganic impurities. Sulphuric acid no longer gave a barium precipitate in water solution. The amount obtained by this method was very small and an exceedingly small part of the original toxic powder. Since the substance obtained in this way still gave good Millon, biuret and xanthoproteic protein reactions, it is fair to say that it was not alkaloidal. The very small amount obtained by this method given to a guinea pig intra-abdominally made the animal sick but did not kill. Either phosphotungstic acid does not precipitate the toxic body or else the amount obtained was less than a fatal dose.

Should the poison contain an alkaloidal body existing as a salt in the acid solution, the possibility of extracting the base with ether or chloroform after the solution had been made alkaline with ammonia, is apparent. This was tried with negative results. To a water solution of colon poison, acid in reaction, ammonia was added, drop by drop, to a slightly alkaline reaction, the mixture shaken with ether, the ether separated and evaporated. The residue remaining was non-toxic. The ammoniacal water solution was next shaken with chloroform, the slightly colored chloroform drawn off and evaporated at low temperature leaving a small amount of a dark, thick semi-liquid, which was not poisonous either as it was or after faintly acidifying with hydrochloric acid. The water solution remaining being still poisonous, it is evident that the toxic part is not an alkaloidal body capable of being extracted directly.

Potassium bismuth iodid in acid solution of the crude soluble poison produces an abundant precipitate, apparently more or less soluble in excess, and soluble in ammoniacal water.

Kowalewsky (36) has shown that uranyl acetate will completely remove from various albuminous fluids every trace of protein giving a biuret reaction while Jacoby (37) and others have used this reagent for the removal of proteins from faintly alkaline solutions. Abel and Ford (38) used it to remove protein from an extract of poisonous fungi. In a slightly alkaline solution of albumen poison, uranium acetate gave an abundant precipitate but not a complete separation as both precipitate and filtrate still gave the Millon and biuret tests and the filtrate, after removal of excess of uranium with a solution of disodium hydrogen phosphate, filtration, evaporation, solution in alcohol and reëvaporation, was still poisonous. In acid solution, the precipitation was complete, the filtrate no longer giving the protein reactions.

Freshly prepared metaphosphoric acid also produced an abundant precipitate but not a complete separation, the filtrate showing both Millon and biuret reactions. Likewise a heavy precipitate is produced by the use of a saturated solution of picric acid but the poison is not in the precipitate which gives only a very poor Millon test after removal of the picric acid and no biuret.

Hofmeister (39) has given a method for introducing iodine into the molecule of egg albumen. This was tried with the poison split from egg albumen. The iodized compound no longer gave either the Millon or biuret reaction, and while it affected animals more or less, they did not die and the symptoms were not those induced by toxin poisoning. The iodine seemed to have entered into chemical combination in the toxin molecule and to have thus changed its characteristics. The iodized body was freely soluble in absolute alcohol and in alkaline water, not in water alone and was precipitated by acid water from alcoholic solution, also on acidifying an alkaline solution. Though it no longer responded to the Millon and biuret reactions, a good test for nitrogen was obtained after fusing with metallic sodium.

An attempt was made to benzoylate the poison by the Schotten-Baumann method using albumin toxin. Practically no

precipitate was obtained. From the filtrate in a part soluble in hot alcohol there were obtained shiny, glistening plates or flat needles which matted together under suction and had much the appearance of some of the fatty acids. These were insoluble in water or very difficultly so if at all, difficultly soluble in cold alcohol, readily in hot. They gave no Millon test, no biuret, no Molisch and contained no nitrogen. After recrystallization from alcohol they melted constantly at 62° . Palmitic acid melts at 62° (Mulliken) and boils at 339° to 350° (Mulliken). A Merck preparation of palmitic acid melted at 62° and boiled at about 345° to 350° . The toxin crystals had not yet boiled at 360° though above 300° there was some decomposition. From the remainder of the filtrate there was obtained from the part soluble in cold alcohol a non-crystallizable body giving both Millon and biuret tests and containing 9.335 per cent of nitrogen, and from the part soluble only in water, likewise a non-crystalline compound, with 9.66 per cent of nitrogen and showing both Millon and biuret tests but not seriously affecting animals in a usual dose.

The nitrogen in a number of the toxins has been determined by Gidley in this laboratory as follows:

TABLE 13.

Percentage of Nitrogen in Protein Poisons.

Source of Poison.	Percentage of Nitrogen in Poison.
Colon bacillus.....	13.49
Typhoid bacillus.....	11.52
Tubercle bacillus.....	11.00
Pyocyaneus.....	10.50
Ruber of Kiel.....	10.495
Subtilis.....	8.12
Megaterium.....	8.595
Proteus Vulgaris.....	10.17
Yellow Sarcine.....	6.145
Egg Albumen (Leach).....	13.74
Serum Albumen.....	10.48
Edestin.....	12.78
Zein.....	10.69
Witte Peptone.....	11.14
De Chapoteaut Peptone.....	12.735

To study the distribution of the nitrogen, determinations were made in both the colon and albumen toxins, of the ammonia nitrogen, the monoamino and diamino nitrogen by the method already described under cleavage with dilute mineral acids.

The following are the results.

TABLE 14.
Distribution of Nitrogen in Protein Poisons.

SOURCE OF POISON.	TOTAL PER CENT N OF POISON.	TOTAL PER CENT N OF ACID EXTRACT.	PER CENT AMMONIA N.	PER CENT MONAMINO N.	PER CENT DIAMINO N.
Colon bacillus.....	13.49	10.185	1.525	6.472	1.753
Egg albumen.....	13.74	11.477	0.745	7.999	1.40

It will be seen that the greater part of the nitrogen is to be found in monoamino combination. From the phosphotungstic filtrates from both the albumen and colon poisons containing the monoamino acids, crystalline bodies were obtained. Judged by the strong Millon test, tyrosin was undoubtedly present, but the crystalline masses were largely leucin, and no tyrosin was obtained in purified form. From the crude crystals, after many and repeated crystallizations what was thought to be leucin was obtained pure, melting at 264° – 265° uncorrected or 269.42° – 270.46° corrected. The crystals were thin plates characteristically grouped and sublimed readily. This will be referred to again later. From another 5 per cent sulphuric acid extract of albumin toxin was obtained a large mass of crystals in characteristic tyrosin-like sheaves and giving a deep Millon reaction. These were undoubtedly tyrosin though at the time no melting point was taken.

Cleavage of the Poisonous Portion with Strong Mineral Acids.

Up to this point every attempt to separate the poisonous body as a chemical unity had been unsuccessful, though each time some new point was added to its chemistry. A still further effort was made. The physiological effect produced by the crude

soluble protein poisons so nearly resembles that of neurin that repeated trials were made to isolate neurin or a neurin-like body. Typhoid toxin, colon toxin, and that from egg albumen and Witte's peptone were all investigated. They were all hydrolyzed with concentrated hydrochloric acid according to Brieger's method (40) and attempts made to form both the mercury and platinum salts. Crystals of various kinds, in exceedingly small amounts, were obtained but none of them poisonous, so that after many repetitions of the process, only one conclusion was possible, namely, that neither neurin nor similar bases were present in these poisons. Before this conclusion was reached, however, owing to the neurin-like physiological effect of the poison in causing death by paralysis of the respiratory centers and in view of the well known antagonistic action of atropin to neurin, the effect of atropin with the poison was tried. Guinea pigs were treated with small doses of atropin sulphate and later given a fatal dose of the Witte peptone toxin, others likewise the colon toxin with control animals treated respectively with poison and atropin. In no case did the atropin itself kill; in all cases the control toxin pigs died. With the peptone toxin previously atropinized guinea pigs, though sick, recovered from the fatal dose. The colon toxin killed the previously atropinized animals, the atropin apparently not protecting. With frogs both toxins made the frogs sick but the peptone toxin did not kill. Thirty milligrams of colon toxin killed, but previously atropinized frogs survived this amount, though 60 mg. killed in spite of atropin. Apparently atropin possesses in some degree a protective action against the protein poisons but not to the extent to which it antagonizes neurin. Parenthetically it may be of interest to state that if guinea pigs receiving fatal doses of poison are immediately and completely etherized and kept under the ether for some 15 minutes, they show no sign of toxin poisoning.

Cleavage for Monoamino Acids. On the whole everything has seemed to point to protein as the nature of the poisonous group itself though undoubtedly protein of much simpler structure than that of the unbroken molecule. Accordingly attention was turned to an investigation and the isolation of certain protein decomposition products by acid hydrolysis, namely, the

monoamino acids. The poisons selected for this work were those from tuberculosis, typhoid and colon germ substances and for comparison that from egg albumen. For the tuberculosis, the poison from 900 grams germ substance was used—296 grams; for the typhoid 100 grams of the poison; for the colon that from 300 grams of germ substance estimated as 61.5 grams; while for the albumen that from 200 grams of the protein was employed yielding, it was estimated, 93 grams toxin. These poisons were hydrolyzed by boiling under a reflux condenser for 14 hours with concentrated hydrochloric acid (sp. gr. 1.19). The nitrogen of each extract was determined by the Kjeldahl method using an aliquot part of each as a sample and giving the following results:

TABLE 15.
Nitrogen of Acid Extract of Poisons.

Source of Poison.	Per Cent N.
Tuberculosis.....	9.895
Typhoid.....	10.38
Colon.....	10.185
Egg Albumen.....	11.477

From this point Fischer's (41) ester method for obtaining the individual monoamino acids was carried out. This method is so well known that it is not necessary to outline it here other than to say that after the amino acids have been produced by cleavage of the protein with concentrated acid, the hydrochlorid of their ethyl esters is formed and later the free esters are separated by distillation in the highest possible vacuum. These are then saponified and the amino acids crystallized and purified. The efficiency of this method depending in large measure upon the vacuum secured, the yields here presented for cleavage of the poisons might have been materially increased with a better vacuum, the highest one possible with the apparatus at hand varying from 20 to 30 mm. The following table shows the results of the distillations of the free esters, both the bath and vapor temperatures being given, the amount of the distillates and the yield of crude crystals after saponification.

TABLE 16.

*Distillation of the Esters of the Monoamino Acids from Protein Poisons.
Tuberculosis Poison.*

FRACTION.	TEMPERATURE OIL BATH.	TEMPERATURE VAPOR.	AMOUNT OF DISTILLATE.	WEIGHT OF CRUDE CRYSTALS.
	<i>degrees</i>	<i>degrees</i>	<i>c.c.</i>	<i>gms.</i>
1.....	40-60	20-40	5	2
2.....	60-80	40-60	5	2
3.....	80-100	60-80	25	16
4.....	100-130	80-100	25	16
5.....	130-160		25	7

No distillate passed over between 20° and 95° inside temperature, or between 110° and 138°.

Typhoid Poison.

1.....	25-60	10-20	12	0.3158
2.....	60-80	20	8	
3.....	80-110	20	2	1.2558
4.....	110-130	95-108	7	
5.....	130-145	108-110	4	6
6.....	145-200	138-185	4	

The yield from the colon poison was exceedingly small due to the fact that at one stage of the process part of the solution was lost.

Colon Poison.

FRACTION	TEMPERATURE OF BATH	TEMPERATURE OF VAPOR	AMOUNT OF DISTILLATE	WEIGHT OF CRUDE CRYSTALS
	<i>degrees</i>	<i>degrees</i>	<i>cc.</i>	<i>gms.</i>
1.....	40-60	28-41	1.5	0.07
2.....	60-80	41-56	1.0	0.06
3.....	80-104	56-84	4.0	2.00
4.....	104-120	84-88	2.0	0.63
5.....	120-160	88-139	5.5	

Albumen Poison.

FRACTION	TEMPERATURE OF BATH	AMOUNT OF DISTILLATE	WEIGHT OF CRUDE CRYSTALS
	<i>degrees</i>	<i>cc.</i>	<i>gms.</i>
1.....	40-60	5	0.2638
2.....	60-80	3	0.3338
3.....	80-100	8	4.000
4.....	100-130	10	6.000
5.....	130-160	7	7.000

After repeated recrystallizations these crude products were obtained in a state of chemical purity. From the tuberculosis poison, fractions 1 and 2 yielded needle shaped crystals, soluble in water and alcohol, sweet to the taste and containing 15.773 per cent of nitrogen, the average of four determinations by the Kjeldahl method. Alanin, $C_3H_7NO_2$, has all these properties and contains 15.73 per cent of nitrogen, thus identifying the crystals as alanin. Fischer, Fränkel and others do not give the melting point for *d*-alanin saying that it is not sharp due to the presence of a mixture of the optically active and the racemic forms. The melting point of the crystals from the tubercle poison varied from 268° to 280° corrected, showing no constant temperature. In fractions 3 and 4, the crystals were beautiful, shiny, satiny plates, sweet to the taste, soluble in water, but almost insoluble in alcohol. These sublimed readily, melted with decomposition, and contained 11.976 per cent of nitrogen (average of eight determinations). These properties and the nitrogen correspond with valin, α -aminoisovalerianic acid, $C_5H_{11}NO_2$ which contains 11.965 per cent nitrogen. Fränkel gives the melting point of valin as 298° corrected; when heated in a closed tube, decomposition taking place at the same time. The valin from the tubercle poison melted as high as 296.28° corrected, but after continued recrystallization the melting point dropped as low as 285° and was never reliable. Whether this was due to a partial racemization on repeated heating is not known. Heated in a closed tube the melting point of the final product was 275.8° - 278.2° . As is well known valin closely resembles leucin in its properties so that it is very difficult to demonstrate the existence of one in the presence of the other. On p. 542, by another method the

presence of leucin in the poisons has been shown but by the Fischer method of ester distillation valin seems to be the one obtained. The presence of leucin was further demonstrated by the fact that from the final residue left after the esters had been distilled crystals of its decomposition product, leucinimid were obtained. This crystallized from dilute alcohol in the form of needles and melted at 295.4° . Cohn gives the melting point of leucinimid as 295° , Fränkel as 262° . From fraction 5 of the tubercle poison a qualitative test only was obtained for phenylalanin, the quantity obtained being too small for complete purification. After evaporation of the ethereal solution of the thick oily ester, according to the method, the ester is saponified by twice evaporating with hydrochloric acid. It is then evaporated with ammonia, dissolved in a small amount of water and poured into a large volume of absolute alcohol, which precipitates the phenylalanin. From this precipitate the qualitative test was obtained, according to Fränkel (42), by dissolving in dilute sulphuric acid and adding an excess of potassium dichromate, producing the characteristic odor of phenylacetaldehyde and showing thus the presence of phenylalanin. From fraction 5, after removal of the phenylalanin, upon saponification with barium hydrate, there were obtained after the barium had been removed, rhombic, hemihedral crystals which had a distinctly sour taste. These, after purification, showed 9.54 per cent of nitrogen, the average of two Kjeldahl determinations, identifying them as glutamic acid, $C_5H_9NO_4$, which has 9.52 per cent of nitrogen. Fränkel gives the melting point of glutamic acid as 202° – 202.5° or quickly heated 213° , with decomposition. The product above obtained melted in an open tube 242° – 245° , in a closed tube 236° – 238° .

From fraction 1 of the typhoid poison was obtained alanin with characteristic properties as described above. These crystals showed 15.633 per cent of nitrogen and melted 267° – 271° . Fractions 2, 3, 4 and 5 contained only valin, which showed 11.932 per cent of nitrogen, the average of four determinations. This melted 287° – 290.6° in an open tube, 278° – 280° in a closed one. From fraction 6, the qualitative test for phenylalanin was obtained as from the tubercle poison.

Owing to the small yield of esters and crystals, fractions 1 and

2 from the colon poison could only be determined qualitatively. Both fractions however showed needle shaped crystals and a sweet taste which in conjunction with the temperature at which their esters distilled indicated alanin. Fractions 3 and 4 gave characteristic valin crystals containing 11.942 per cent of nitrogen and melting at 283.4° – 285° in an open tube or 274° – 277° in a closed tube. Phenylalanin was obtained qualitatively from this as from the two preceding poisons. After its extraction from fraction 5, and after saponification with barium hydrate and its removal, crystals in the form of rhombic plates and prisms, insoluble in alcohol, were obtained. These correspond with those of aspartic acid, and as the quantity was not sufficient for purification by recrystallization, the copper salt was formed with copper acetate. This was obtained in the form of needles, very difficultly soluble in cold water, difficultly in hot, which again correspond with the properties of aspartic acid.

When the crystals from the fractions obtained from the albumen poison, were examined the result was not different. Fractions 1 and 2 produced characteristic alanin crystals with 11.75 per cent of nitrogen, the average of four determinations. The melting point was 277° – 279.6° . Valin, with form and properties as already given was obtained from both fractions 3 and 4, containing 11.935 per cent of nitrogen, and showing a melting point of 282° – 286.4° in an open tube, and of 279° – 283° in a closed tube. Likewise from fraction 5, the heavy oil of phenylalanin ethyl ester was obtained and from this as in the other cases the qualitative test for phenylalanin by the production of phenylacetaldehyde. The remaining portion of fraction 5 yielded the same rhombic plates and prisms as described under the colon fractions and which are like those of aspartic acid properly obtained at this point if present. The copper salt was again formed, the same needles, very difficultly soluble in cold water, difficultly in hot, being obtained. The amount of crystals was too small for further identification.

From this it will be seen that monoamino acids are obtained from the protein poisons after hydrolysis with strong acid. It is not claimed that these are the only monoamino acids present or that all of these have been sufficiently identified, but in consideration of the fact that those discussed were found in the

proper fraction according to Fischer's separation and according to the boiling points of their esters, that the crystalline form and qualitative properties corresponded, and that, when it could be determined, the percentage of nitrogen was close to the theoretical it seems fair to conclude that the following tabulation is not far from correct.

TABLE 17.
Monoamino Acids of the Protein Poisons.

TUBERCULOSIS POISON.	TYPHOID POISON.	COLON POISON.	ALBUMEN POISON.
Alanin	Alanin	Alanin	Alanin
Valin	Valin	Valin	Valin
Phenylalanin	Phenylalanin	Phenylalanin	Phenylalanin
Glutamic Acid			
Leucinimid		Aspartic acid	Aspartic acid

This is sufficient to establish the point for the proof of which the method was employed, that is the protein nature of the poisonous group of the protein molecule. Attention is called also to the comparative simplicity of the group and to the great similarity of acids obtained from the different poisons. This accords well with the great similarity and non-specificity of their physiological action.

It is interesting that the final residue left after distillation of the esters gives still a very intense Millon reaction, which cannot be ascribed to the presence of tyrosin.

To recapitulate briefly, the bacterial cellular substances obtained in large quantity freed from non-poisonous alcohol-ether extractives, are of complex but definite protein composition and are highly poisonous whether derived from pathogenic or non-pathogenic bacteria. They respond to all the protein color reactions. Physical solvents scarcely affect them but they are partially digested by pepsin and trypsin with a lessening of their poisonous properties. Both dilute and strong mineral acids effect definite cleavages, which show the chemical nature of the whole protein, but do not separate the poisonous group in a free form. This is accomplished by hydrolysis with

dilute alkali, best in alcoholic solution, which leaves the non-poisonous portion undissolved. The process is accompanied by the production and loss of ammonia. The non-poisonous portion of the cellular proteins shows most of the protein color reactions, contains all the carbohydrate of the unsplit molecule, most of the phosphorus and is the specific part of the cell, the part that immunizes and sensitizes. The poisonous portion, freely soluble in absolute alcohol, shows all the protein color reactions except the carbohydrate test. It is highly poisonous, killing in less than an hour, but has no specificity in its action, the symptoms being identical whatever the protein from which the poison is taken. It has not been possible to obtain any poisonous base or to isolate the toxin as a chemical unity, but from the monoamino acids obtained after its hydrolysis with concentrated acid it is concluded that the poison itself is protein in nature, though simpler in structure than the complex proteins of the bacterial cells themselves.

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